THE EMBO JOURNAL

Manuscript EMBO-2009-71567

Endocytosis Restricts Arabidopsis KNOLLE Syntaxin To The Cell Division Plane During Late Cytokinesis

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Review timeline:

Submission date:	09 June 2009
1st Editorial Decision:	10 July 2009
1st Revision received:	17 October 2009
2nd Editorial Decision:	04 November 2009
Accepted:	06 November 2009

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 10 July 2009

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has now been seen by three referees whose comments to the authors are shown below. As you will see while the referees consider the study as being interesting in principle, referees 2 and 3 feel strongly that certain aspects of the study need to be strengthened before they can support publication of the study here. A major concern is the conclusiveness of the FLIP and FRAP experiments as GFP-KNOLLE behaves differently from endogenous KNOLLE in terms of its localisation even though it does complement the knolle mutant phenotype. Referee 3 suggests addressing this issue by analysing the behaviour of control markers to validate the findings. Furthermore, referees 2 and 3 feel that the link between sterols and endocytosis in the restriction of KNOLLE to the cell plate remains somewhat weak. Taking together all these issues it becomes clear that the strength of the experimental evidence presented needs to be increased considerably. We would thus be able to consider a revised manuscript if you can address the concerns put forward by all three referees to their satisfaction and if you can strengthen the conclusiveness of the data considerably along the lines pointed out in detail.

I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript as well as on the final assessment by the referees.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

Referee #1 (Remarks to the Author):

The manuscript presented by Boutté and co-workers describes the analysis of molecular mechanisms that restrict subcellular localization of the syntaxin KNOLLE to the plane of division during late cytokinesis in Arabidopsis. The authors use a combination of genetics, pharmacology, immunocytochemistry and cell biology to demonstrate that endocytosis is the process that predominantly restricts KNOLLE localization. Lateral diffusion of KNOLLE from the cell plate to the adjacent plasma membrane is compensated for by endocytotic cycling that is dependent on sterols, clathrin and dynamin-related protein 1A.

The present manuscript addresses a fundamental (and largely unresolved) question in biology, namely how cellular polarization is established and maintained. A striking example of cellular polarization is the formation of the cell plate during cytokinesis. This process, which despite some differences is conserved in higher eukaryotes, involves in Arabidopsis the polarized localization of the KNOLLE syntaxin. Owing to its general impact and novelty, this manuscript is suited for a broad readership.

The authors elegantly combined genetic analysis, pharmacological interference and cell biological approaches in their work. Results from the various types of experiments nicely supplement each other and adequately support the claims of the authors. All experiments are well documented and include the appropriate controls. The Figures are of high quality and contain all relevant information such as labels and size bars. Informative Supplemental information complements the main manuscript (for example, I highly appreciate showing the full-size Western blots in Supplemental Figure 4). All in all this is an excellent manuscript that meets the highest standards, and I strongly recommend its publication. I have only a few suggestions for improvement:

1.) In the "Discussion" (actually, the two paragraphs considered to be the Discussion - see also point 8 below), the authors fail to integrate their findings into a broader context of cell polarization/lateral membrane compartmentalization. For example, endocytosis was recently shown to mediate and maintain the cellular polarization of auxin efflux carriers of the PIN family (another prominent example of cellular polarization in plant cells; Nature 456, 962-966 (2008)). The authors may wish to discuss the commonalties and differences, respectively, between polar PIN and KNOLLE localization. The authors may likewise consider integrating and discussing their findings in the even broader context of general mechanisms leading to membrane compartmentalization at the nano, micro and macro level (e.g. as recently reviewed in Curr. Opin. Plant Biol. 11, 632-640 (2008)).

2.) It is not clearly introduced whether sterol biosynthesis mutants cpi1-1 and smt1 have any recognizable cytokinetic defects. Although the present manuscript nicely demonstrates that endocytosis confers restriction of KNOLLE localization to the plane of division, it does not become clear whether this restriction to the focal plane is a requirement for proper cytokinesis (and thus, plant development). Although this aspect is briefly mentioned in the Discussion (page 12, line 1), it should be brought up much earlier.

3.) Page 5, line 2: The authors state that "KNOLLE commonly localized to lateral plasma membranes", although quantitative analysis shown in Figure 4t suggests that only 40% of the cpi1-1 cells showed this phenomenon.

4.) Page 10, lines 4-5For the broader readerhip it would be useful to state which role Rab11-like RabA2a and Rab5 GEF VPS9a have and why exactly these markers were selected for analysis.

5.) Page 15: In the Methods section promoters of all marker constructs should be indicated and a

consistent nomenclature for transcriptional fusion either : or :: being used
6.) Page 15: Was really 1 M MES used in the liquid MS medium?

7.) In the context of the experiments shown in Figure 3 it would be interesting to see how GFP-KNOLLE fluorescence recovery would look like if the entire plane of division (and not only part of it as shown in panels a and b) would be bleached (either in the presence or absence of energy inhibitors). I strongly encourage the authors to add this experiment (if they do not have the data anyway) to Figure 3.

8.) It is quite obvious from the general (non-narrative) style, the lack of division in Introduction, Results and discussion and the format of the references that this manuscript was originally written and formatted for another journal. The authors should at least take the effort to subdivide the manuscript into the common sections and to re-format cited references and the bibliography according to the EMBO style when they submit the manuscript to this journal :). Overall, each section (especially Intro and Discussion) would benefit from some expansion.

9.) Finally, for a broad readership, the title should contain a word such as "plant" or "Arabidopsis" to allow readers to judge what kind of organism this story is about.

Referee #2 (Remarks to the Author):

The manuscript investigates the role and regulation of KNOLLE synatxin in plant cell division. KNOLLE is known to be required for the abscission step of cytokinesis, thus understanding it dynamics and regulation during cell division is of obvious interest. The authors investigate the observation that treatment of cells with drugs altering sterol biosynthesis causes lateral diffusion of KNOLLE from cell plate to lateral plasma membrane. It is and interesting observation, although, authors never really explained or demonstrated how sterols may cause that. Instead, authors switch to investigating role of endocytosis in regulating KNOLLE localization to the plain of cell division. They show that inhibition of endocytosis clearly results in increased diffusion of KNOLLE to the lateral membrane. The final conclusion suggests that increased endocytosis of KNOLLE at the lateral membrane is what causes the restricted distribution of KNOLLE. It is a nice hypothesis that is supported but not proved by shown work. For study to be more convincing, few additional experiments need to be done. First, is increased endocytosis at lateral membrane specific to KNOLLE? Authors should check other plasma membrane proteins, including the ones that are and are not enriched ant the plane of cell division. Is decreased endocytosis of KNOLLE at plane of division or even targeted exocytosis of KNOLLE to plane of division also play a role in its localization? Is KNOLLE targeted to plane of division via transport through RabA2a? Data seem to suggest that. Completion of all these experiments would greatly enhance the manuscript and would allow authors to provide a more clear and comprehensive model. Finally, the validity of FLIP and FRAP experiments are questionable, since GFP-KNOLLE localizes quite differently from endogenous KNOLLE. Thus, I am not sure how relevant is the data derived using GFP-KNOLLE.

Referee #3 (Remarks to the Author):

The manuscript by Boutte et al. entitle " Endocytosis Restricts KNOLLE Syntaxin To The Plane Of Cell Division During Late Cytokinesis " describes the involvement of sterols and endocytosis in restricting KNOLLE syntaxin to the cell plate. The precised subcellular distribution of vesicles derived from trans-golgi network and endosomes to the newly formed cell plate is a central question in cell biology especially to understand cytokinesis. The present work used state of the art approaches (FRAP, FLIP) and materials (mutants and markers) to unravel this important question. The authors provided two important piece of data explaining the mechanism of syntaxin restriction to the cell plate. First, sterols cololocalize partially with KNOLLE and are important for restricting the syntaxin to the cell plate. Second, KNOLLE is able to move by lateral diffusion from the new membrane to lateral membranes and this movement is prevented by endocytosis. These conclusions are backed up by rationally designed experiments and high quality data.

I have however few comments about some of the conclusion raised by the authors:

1-The main concern I have concerns the link between sterol and endocytosis in the restriction of KNOLLE to the cell plate. The authors claim in the abstract that the localization of KNOLLE in the cell division plane is maintained by sterol-dependent endocytosis (p3). This is not supported by the data. There is no doubt that sterols and endocytosis are involved but there is no evidence that the clathrin and dynamin- related endocytosis require or involved sterols. It is a very important statement that needs to be clarified. The authors showed that sterols partially colocalized with early endosome. How do these markers behave (number and size of vesicules) in absence of sterols (with fenpropimorph treatment for instance)? The FM4-64 dye labels very quickly ealy endosome population and could therefore be used to monitor endocytosis in sterol depleted cells with time course experiment for instance.
An accessory question is why the manuscript title does not refer to sterols and conversely why the objectives of the work at the end of introduction refers only to sterols? From the reader point of view, the relationship between sterols and endocytosis remains a bit confused along the manuscript and needs to be clarified.

2- The partial colocalization of sterol dye filipin with different endomembrane markers is an important experiment linking sterols to early endosomes. The authors state that there is a sterol gradient throughout the endomembrane system (last sentence p6). I do not think this claim is entirely justified since the data showed that there is a gradient of colocalization between endomembrane markers and filipin. According to the experiment, the different classes of vesicules are either stained or not i.e containing sterol or not. There is no evidence of gradient of lipids per se. The authors need to rephrase their conclusions.
I am also quite surprised with the figures. It is difficult to conceive that some KNOLLE vesicules do have sterols and some do not. I agree that sterol content might vary among the population of vesicules (before and after fusion at the plasmamembrane for instance) but it is surprising that 60% of KNOLLE vesicules are not stained with filipin (the highest relative value among markers. What is known about the binding dynamic and specificty of filipin with sterols?
Such result might very well reflects the low resolution of the colocalization experiment or a bias in filipin and/or KNOLLE staining. It is not clear to me whether the figures relate to 2D analysis or 3D reconstruction. A single 3D analysis with one marker at a better resolution would definetely validate the approach.

3- In the same line, the authors stated that KNOLLE doe not seem to be associated with sterol in lipid rafts. I agree with the authors that KNOLLE might interact with sterol outside lipid rafts. However Fig.4q shows some discrepancies between the two fractions R4 and R8. Indeed R8 does not show a relative enrichment in KNOLLE amount but the intensity of the band is clearly much higher compared to R4. Since this kind of experiment is difficult to normalize quantitatively, it raises some doubt on the actual interaction between KNOLLE and lipid rafts. If R8 is taken from granted, KNOLLE is clearly in an intermediate binding situation compared to the lipid raft marker PM-ATPase and a non raft protein like SMT1.
The fact that KNOLLE is not associated with lipid raft but is still altered by sterol depletion raises the question of how KNOLLE and sterols interact? A recent work in yeast (Klemm et al. J. Cell Biol. 2009, 185, 601-12) demonstrated that TGN is a dispatching platform for sterol rich domains (rafts) and their corresponding proteins. If the model applies to plant, one would expect that the absence of KNOLLE from rafts should lead to relative insensitivity of KN dynamics to sterol depletion unless it is indirectly mediated by endocytosis. The fact that sterol depletion alters dynamin subcellular distribution (ref 31) suggests that this might be case and would thus provide a possible answer to my first concern.

4- The measurement of lateral diffusion of KNOLLE-GFP is a very important experiment since it demonstrates that KNOLLE-GFP diffuses in lateral membranes after cytokinesis and rules out the role of sterols in this process. I am convinced with the FRAP and FLIP experiment which are of high standarts. However the use of KNOLLE-GFP protein fusion raises some concern. Contrary to the endogenous KNOLLE protein, KNOLLE-GFP fusion labels not only the cell plate but also the other plasmamembranes. The authors exploited this property for their diffusion experiment but did not comment the reason of the retention of KN-GFP in plasmamembranes. It can be a major concern since the whole point of the study is to unravel the mechanism restricting KNOLLE to the cell plate. One might very well assume that KN-GFP endocytosis is altered because of the new properties of the fusion protein. So concluding to the absence of sterol effect in lateral diffusion based on the used

of a GFP construct which has different membrane retention properties is very questionable. What is the diffusion rate of other rab markers (rabA2a) or FM4-64 in presence or absence of fen?

1st Revision - authors' response 17 October 2009

Point-by-point author response to reviewers'comments (rebuttal)

We thank all three reviewers for their constructive comments which we feel have helped to improve our manuscript.

Before we address all specific points in detail, we give an overview over the main points raised by the reviewers and editor which we now address by including additional data:

One concern of reviewer 2 and 3 was the conclusiveness of the FLIP and FRAP experiments as GFP-KNOLLE behaved differently from endogenous KNOLLE in terms of its localization even though it does complement the knolle mutant phenotype. Referee 3 suggested addressing this issue by analysing the behaviour of control markers to validate the findings.

1) We now include additional FRAP analyses of four marker molecules in the wild-type and cpi1-1 mutant background as new Supplementary Figure 4. The results support our previous conclusion that lateral diffusion per se does not appear to be enhanced in the cpi1-1 mutant (referee 2 and 3).

2) We additionally include analysis of three markers under conditions of energy inhibition of which native KNOLLE protein is the only one showing lateral diffusion to the plasma membrane (referee 2 and 3) as new Supplementary Figure 5.

3) We also include additional FRAP data requested by referee 1 as Figure 5I-K and Supplementary Figure 4A-C.

Referees 2 and 3 felt that the link between sterols and endocytosis in the restriction of KNOLLE to the cell plate remained somewhat weak. We now provide five different lines of evidence to further strengthen this link:

4) We report strong co-localization of KNOLLE and CLATHRIN-LIGHT-CHAIN-GFP at the late cell plate and in endomembrane compartments in Supplementary Figure 7V-X.

5) We demonstrate that sterols localize to the cell plate, cell division plane and compartments of the early endocytic pathway such as the TGN as well as to multivesicular bodies, employing transmission electron microscopy visualization of filipin-sterol deformations at these compartments in the new Figure 3.

6) We report that during internalization at the end of cytokinesis KNOLLE is more strongly retained at the cell-division plane of the cpi1-1 mutant compared to wild type (both under CHX and BFA treatment), suggesting a defect in KNOLLE removal from the cell-division plane upon interference with sterol composition (new Figure 6).

7) We show that the cpi1-1 mutation reduces sterol localization with KNOLLE and at early and late endosomal compartments, by employing filipin-sterol fluorescence co-localization with eight different compartment (new Supplementary Figure 6).

8) In addition to similar KNOLLE mis-localisation in the dynamin-related protein1a and cpi1-1 mutants, we now report a strong synergistic genetic interaction between DYNAMIN-RELATED PROTEIN1 and CPI1 revealed by synthetic seedling lethality of a drp1a;cpi1-1 double mutant (new Figure 8).

Detailed responses:

Referee #1 (Remarks to the Author):

[...] All in all this is an excellent manuscript that meets the highest standards, and I strongly recommend its publication. I have only a few suggestions for improvement:

Author response: Thank you for the encouraging comments.

1.) In the "Discussion" (actually, the two paragraphs considered to be the Discussion - see also point 8 below), the authors fail to integrate their findings into a broader context of cell polarization/lateral membrane compartmentalization. For example, endocytosis was recently shown to mediate and maintain the cellular polarization of auxin efflux carriers of the PIN family (another prominent example of cellular polarization in plant cells; Nature 456, 962-966 (2008)). The authors may wish to discuss the commonalties and differences, respectively, between polar PIN and KNOLLE localization. The authors may likewise consider integrating and discussing their findings in the even broader context of general mechanisms leading to membrane compartmentalization at the nano, micro and macro level (e.g. as recently reviewed in Curr. Opin. Plant Biol. 11, 632-640 (2008)).

Author response: We have integrated both references. We discuss "the commonalities and differences between polar PIN and KNOLLE localization" to quite some extent at the beginning of the Discussion on page 18. The Zappel & Panstruga 2008 (Curr. Opin. Plant Biol. 11, 632-640) paper has been integrated in the Results section on page 9. However, we do not feel that a more detailed discussion of nano, micro and macrodomains is appropriate here at present, because there is hardly any data available on the in vivo existence and in vivo functionality of such potentially sterol- and sphingolipid enriched domains in plants which mention in the Results on page 9.

2.) It is not clearly introduced whether sterol biosynthesis mutants cpi1-1 and smt1 have any recognizable cytokinetic defects. Although the present manuscript nicely demonstrates that endocytosis confers restriction of KNOLLE localization to the plane of division, it does not become clear whether this restriction to the focal plane is a requirement for proper cytokinesis (and thus, plant development). Although this aspect is briefly mentioned in the Discussion (page 12, line 1), it should be brought up much earlier.

Author response: We had cited the two papers by Schrick et al. 2004 and our own lab (Men at al. 2008, Nature Cell Biology 10: 238-244) in which these defects are described in the previous manuscript version, but we have now extended the description in the Introduction on page 4 to clarify this point, according to the reviewers suggestion.

3.) Page 5, line 2: The authors state that "KNOLLE commonly localized to lateral plasma membranes", although quantitative analysis shown in Figure 4t suggests that only 40% of the cpi1-1 cells showed this phenomenon.

Author response: We now specify the amount of cells as "43%" in the text Results on page 4.

4.) Page 10, lines 4-5For the broader readerhip it would be useful to state which role Rab11-like RabA2a and Rab5 GEF VPS9a have and why exactly these markers were selected for analysis.

Author response: Thank you for the suggestion. We now clarify this in detail within the results section on page 16.

5.) Page 15: In the Methods section promoters of all marker constructs should be indicated and a consistent nomenclature for transcriptional fusion either : or :: being used

Author response: We now keep the nomenclature consistent, according to the suggestion of this reviewer.

6.) *Page 15: Was really 1 M MES used in the liquid MS medium?*

Author response: This was indeed an error. It has been corrected to 1x MS

7.) In the context of the experiments shown in Figure 3 it would be interesting to see how GFP-KNOLLE fluorescence recovery would look like if the entire plane of division (and not only part of it as shown in panels a and b) would be bleached (either in the presence or absence of energy inhibitors). I strongly encourage the authors to add this experiment (if they do not have the data anyway) to Figure 3.

Author response: We have performed the requested experiments in wild-type and cpi1-1 mutant background with CHX and energy inhibitors. The new data has been included as Figure 5I-K and Supplementary Figure 4A-C. Consistent with our previous findings, the results reveal a very similar behavior with no significant difference between the wild type and cpi1-1. The recovery, as expected, is somewhat slower than if only a 2 micrometre region is spot bleached such as in Figures 5A and B. We also include the FLIP curves for loss of fluorescence from the plasma membrane which show that GFP-KNOLLE can also diffuse from the lateral membrane to the cell plate.

8.) It is quite obvious from the general (non-narrative) style, the lack of division in Introduction, Results and discussion and the format of the references that this manuscript was originally written and formatted for another journal. The authors should at least take the effort to subdivide the manuscript into the common sections and to re-format cited references and the bibliography according to the EMBO style when they submit the manuscript to this journal :). Overall, each section (especially Intro and Discussion) would benefit from some expansion.

Author response: We had made use of the direct manuscript transfer service that Nature Publishing Group (NPG) offers to authors from one journal to another. The different journals have, however, somewhat different formats. We have reformatted the manuscript to EMBO Journal style, have expanded the introduction and discussion sections and included additional figures.

9.) Finally, for a broad readership, the title should contain a word such as "plant" or "Arabidopsis" to allow readers to judge what kind of organism this story is about.

Author response: This has been done.

Referee #2 (Remarks to the Author):

The manuscript investigates the role and regulation of KNOLLE synatxin in plant cell division. KNOLLE is known to be required for the abscission step of cytokinesis, thus understanding it dynamics and regulation during cell division is of obvious interest. The authors investigate the observation that treatment of cells with drugs altering sterol biosynthesis causes lateral diffusion of KNOLLE from cell plate to lateral plasma membrane.

Author response: Thank you.

It is and interesting observation, although, authors never really explained or demonstrated how sterols may cause that.

Author response: To elucidate the exact molecular mechanisms of how different sterols interact with a specific protein molecule in the plasma membrane causing the observed effects would require several years of additional work. We feel this lies beyond the scope of the present study.

Instead, authors switch to investigating role of endocytosis in regulating KNOLLE localization to the plain of cell division. They show that inhibition of endocytosis clearly results in increased diffusion of KNOLLE to the lateral membrane.The final conclusion suggests that increased endocytosis of KNOLLE at the lateral membrane is what causes the restricted distribution of KNOLLE. It is a nice hypothesis that is supported but not proved by shown work.

Author response: The second last sentence suggest to us that we may not have stated this clearly enough in the previous version of the manuscript and might have been mis-understood. We do NOT want to specifically state that "increased endocytosis of KNOLLE at the lateral membrane is what causes the restricted distribution of KNOLLE.". In fact DYNAMIN-LIKE PROTEIN1A (DRP1A)-

GFP strongly localizes to the cell division plane and somewhat weaker, but significantly, to the lateral plasma membranes. The drp1a mutants analyzed, however, show an extremely strong lateral and/or overall plasma membrane localization of KNOLLE. Hence, it could be both reduced endocytosis from the plane of cell division and/or the lateral membrane, which causes these defects by allowing for more KNOLLE to diffuse over to the lateral membrane.

We are glad that this reviewer raised this point and now specify in the discussion on page 20 that

"Two not necessarily mutually exclusive scenarios can be envisaged as to how DRP1A and clathrin-mediated endocytosis could restrict KNOLLE localization to the plane of cell division. Since both KNOLLE and DRP1A accumulate at the leading edge of the cell plate, DRP1A may already be involved in recycling of KNOLLE at this stage. However, weaker DRP1A accumulation is also observed at the (lateral) plasma membrane (cf. Figure 1N), and this subpopulation of DRP1A foci may additionally contribute to restrict lateral diffusion of KNOLLE."

Furthermore, a contribution of endocytosis in the plane of cell division is indicated by the new data included in Figure 6J-N, where we see that KNOLLE remains more strongly at the cell-division plane of cpi1-1 mutant cells treated with BFA and CHX for 60 min, when compared to the wild type under the same conditions of treatment. This is described in the Results on page 14.

For study to be more convincing, few additional experiments need to be done. First, is increased endocytosis at lateral membrane specific to KNOLLE? Authors should check other plasma membrane proteins, including the ones that are and are not enriched ant the plane of cell division.

Author response: As mentioned above, we did not intend to state that we think that there is an increased endocytosis at the lateral membrane.

Nevertheless, we agree with the reviewer that it is helpful to see how other proteins behave under energy inhibition i.e. as to whether they also diffuse to the lateral membrane. This has not been very conclusive to evaluate for proteins localized all over the plasma membrane to start with (e.g. the small plasma membrane protein EGFP-LTI6a which we, instead, analysed in FRAP studies, see below). However, it was possible to see how proteins that are mostly localized to the plane of cell division at the end of cytokinesis, such as (GFP)-KNOLLE, PIN2-(EGFP), DRP1A-GFP and YFP-RAB-A2a, behave and we include additional data on these proteins in Supplementary Figure 5A-D'.

Consistent with the much slower lateral diffusion of PIN2-EGFP in the plane of cell division (Men at al. 2008)and at the lateral membrane, when compared to GFP-KNOLLE (this study, see below), we did not observe significant lateral membrane labeling for endogenous PIN2 detected by specific antibodies nor PIN2-EGFP after 30 min incubation with CHX and energy inhibitors (new data Supplementary Figure 5G-L). We also did not observe a relocation of functional DYNAMIN-RELATED PROTEIN1A (DRP1A)-GFP to the lateral plasma membrane under conditions of either CHX treatment or CHX treatment plus energy inhibition (new data Supplementary Figure 5M-U). The small GTPase RAB-A2a fused to YFP rapidly disappeared from the plane of cell division upon treatment with energy inhibitors (new data Supplementary Figure 5V-D'). Hence, we conclude that various membrane associated or transmembrane proteins may display divserse responses with respect to their localization at the lateral plasma membrane when endocytosis is inhibited by energy deprivation. Nevertheless, the proteins tested KNOLLE displayed the fastest and most obvious lateral relocation (new data Supplementary Figure 5). This is described in the Results section on page 13 and discussed on page 19.

Is decreased endocytosis of KNOLLE at plane of division or even targeted exocytosis of KNOLLE to plane of division also play a role in its localization?

Author response: The reviewer indicates correctly that it is likely the balance between endocytosis of KNOLLE from the plane of cell division and from the lateral membrane as well as its exocytosis which plays a role in KNOLLE localization. It has previously been shown that there is a very strong contribution of targeted KNOLLE exocytosis at the cell plate (Reichardt et al. 2007. Curr Biol 17: 2047-2053).
Therefore, we have now focused on endocytosis and lateral diffusion which we clearly show to contribute to KNOLLE localization at the end of cytokinesis. The reviewer may consider that prior

to cell-plate fusion we never observed KNOLLE at the plasma membrane, except for the cell-plate and endogenous endomembrane compartments (cf. Supplementary Figure 1A-R) hundreds of cells examined for the localization of native, endogenous KNOLLE protein in any of the wild-types and mutants analysed here. Hence, the defects we observe are highly unlikely due mis-tartgeted exocytosis.

Is KNOLLE targeted to plane of division via transport through RabA2a? Data seem to suggest that.

Author response: Consistent with the reviewerís suggestion we do reproducibly observe a weaker KNOLLE signal in the plane of cell division in immunolabelling experiments of plants expressing the dominant-negative rab-A2aN125I mutant version (Supplementary Figure 7). To find out whether this is due to reduced secretory targeting or recycling from the cell plate or plasma membrane would be the task for an additional study. This is especially difficult as multiple (up to quadrupole or pintuple) mutants in different RAB-A GTPases would need to be generated to correctly address this point. We feel that it is not the topic of this work to specifically elucidate the function of RAB-A GTPases. We also do not think that this point is of major importance to this manuscript, since the main aim was to see whether we could find a link between the lateral mis-localisation defects observed in sterol biosynthesis mutants and mutants defective in components in the endocytic machinery.

Completion of all these experiments would greatly enhance the manuscript and would allow authors to provide a more clear and comprehensive model. Finally, the validity of FLIP and FRAP experiments are questionable, since GFP-KNOLLE localizes quite differently from endogenous KNOLLE. Thus, I am not sure how relevant is the data derived using GFP-KNOLLE.

Author response: The reviewer correctly points out that one cannot be absolutely certain that a GFP fusion protein exhibits exactly the same dynamics as the endogenous protein even if, as in our case, it completely rescues the mutant phenotype. However, we would like to ask the reviewer to consider the following points:

1) In contrast to most other studies in the Arabidopsis field (including those published in EMBO J.), we provide data for a functional GFP(-KNOLLE) fusion (Figure 5A-N) and the endogenous KNOLLE protein (Figure 5O-Q), demonstrating that also the endogenous KNOLLE protein does laterally diffuse under conditions of energy inhibition (Figure 5O-Q).

2) We fully agree with the reviewer that we cannot be certain that the dynamics and speed of lateral diffusion are exactly the same for endogenous KNOLLE and GFP-KNOLLE. Note, however, that we also did not claim this in the previous or current version of the manuscript. Nevertheless, we feel that our data does make a relevant point, namely that GFP-KNOLLE as well as endogenous KNOLLE display lateral diffusion from the plane of cell division to the plasma membrane.

3) Finally, one additional observation from our FRAP analyses was that we do not find enhanced lateral diffusion of GFP-KNOLLE in the cpi1-1 mutant background, when compared to wild type. Reviewer 3 suggested estimating whether this applies also for other markers which should also help to address this similar point of reviewer 2.

According to the reviewers' request, we now include FRAP analyses from a region at the lateral plasma membrane for FM4-64, GFP-KNOLLE, PIN2-EGFP and EGFP-LTI6A under CHX treatment and energy inhibition in wild-type and cpi1-1 mutant background as additional data in Supplementary Figure 4E-P. None of these molecules covering a large range of lateral diffusion rates showed a clearly enhanced lateral diffusion in the cpi1-1 mutant background (integrated into the Results on page 13). These findings are further consistent with our previous interpretations and we conclude that lateral diffusion, per se, is not likely to be generally enhanced in the cpi1-1 mutant. We feel that the data from the different molecules including the GFP-KNOLLE fusion protein has added some relevant information.

Referee #3 (Remarks to the Author):

[...] These conclusions are backed up by rationally designed experiments and high quality data.

Author response: Thank you for these encouraging comments.

I have however few comments about some of the conclusion raised by the authors:

1-The main concern I have concerns the link between sterol and endocytosis in the restriction of KNOLLE to the cell plate. The authors claim in the abstract that the localization of KNOLLE in the cell division plane is maintained by sterol-dependent endocytosis (p3). This is not supported by the data.

Author response: We have previously shown that the cpi1-1 mutant employed in this study is defective in uptake of the endocytic tracer FM4-64, internalization of endogenous PIN2 and PIN2-EGFP proteins just after cell division (Men at al. 2008). We have now introduced a sentence to already clarify this in the introduction on page 5:

"Interestingly, the cpi1-1 mutant has been found to be defective in the internalization of the endocytic tracer FM4-64 and the Arabidopsis PIN2 protein from plasma membranes of dividing and post-cytokinetic root epidermal cells (Men et al, 2008)."

Nevertheless, we now also include further experiments in which we localize the endogenous KNOLLE protein in wild-type and cpi1-1 mutant roots under treatment with the protein biosynthesis inhibitor cycloheximide, to disrupt the biosynthetic secretory supply of KNOLLE, and under treatment with the ARF-GEF inhibitor brefeldin A which inhibits endocytic recycling in Arabidopsis roots. These experiments show that KNOLLE is more strongly retained in the plane of cell division after 60 min of treatment with 10 uM BFA in the cpi1-1 mutant background when compared to the wild type (new data Figure 6J-N). These findings very strongly suggest that endocytosis of both PIN2 and KNOLLE is reduced in cpi1-1 mutant roots.

There is no doubt that sterols and endocytosis are involved but there is no evidence that the clathrin and dynamin- related endocytosis require or involved sterols.

Author response: The reviewer may consider that Sebastian Bednarek's group recently published that the residence time of both DRP1A-GFP and CLATHRIN-LIGHT-CHAIN (CLC)-GFP at the plasma membrane in elongating cells is prolonged by treatment with the sterol biosynthesis inhibitor fenpropimorphand that this was included in the discussion of our previous manuscript version (Konopka and Bednarek 2008. Plant Physiol 147: 1590-1602; Konopka et al. Plant Cell 20: 1363-1380). Fenpropimorph mainly, but not solely, targets the CPI1 and SMT proteins.

In this study, we demonstrate defects in KNOLLE localization in cpi1-1 and different smt1 mutant backgrounds as well as under fenpropimorph treatment, and have added further data on the we co-localization of KNOLLE and CLC-GFP (new data in Supplementary Figure 7V-X).
Moreover, as further evidence strengthening the point of an interaction between sterols and dynamin-like protein interaction, we now include analyses of a dynamin-related protein1A;cpi1-1 double mutant which displays synthetic seedling lethality, revealing a synergistic interaction between the DRP1A and CPI1 genes. This new data is displayed in Figure 8, included in the Results on page 17 and in the Discussion on page 20.

It is a very important statement that needs to be clarified. The authors showed that sterols partially colocalized with early endosome. How do these markers behave (number and size of vesicules) in absence of sterols (with fenpropimorph treatment for instance)?

Author response: The reviewer asks "how these markers would behave (numbers and size of vesicules) in absence of sterols (with fempropimorph) treatment for instance". Fenpropimorph treatment primarily, but not only, targets cyclopropylsterol isomerisation and CPI1is the only cyclopropylsterol isomerase known in Arabidopsis.

For the sake of consistency and specificity, we addressed the behaviour eight different endomembrane system markers including KNOLLE would behave in the cpi1-1 mutant backround in co-labelling studies with filipin-sterol fluorescence. We already had this data, otherwise it would not have been possible to perform this in the time span of three months. We now include this new

data as Supplementary Figure 6A-Q'. Our results show that in the cpi1-1 mutant co-labelling of filipin-sterol fluorescence is significantly reduced with markers residing in early endosomal/trans-Golgi Network compartments such as KNOLLE, YFP-RAB-A-2a, ARF1-EGFP and markers for the prevacuolar compartments/multivesicular bodies such as RAB-F1-GFP, GFP-RAB-F2b Supplementary Figure 6Q'.The experiments are described in the Results as paragraph "Sterol localization to KNOLLE-positive, TGN and MVB compartments is altered in the cpi1-1 mutant" on page 14-15. Our findings clearly show that sterol localization is reduced in compartments of the endocytic pathway in the cpi1-1 mutant.

Just to clarify this point: fenpropimorph treatment, similar to mutation of e.g. the Arabidopsis CPI1 and SMT1 genes, does not primarily cause sterol depletion as indicated by this reviewer but a change in the sterol profile to precursor sterols such as cyclopropylsterols and cholesterol, respectively (Schrick et al. Plant J 31: 61-73. He et al. 2003. Plant Physiol 131: 1258-1269; Men et al. 2008). Sterol depletion results in early lethality as for example recently shown for the cycloartenol synthase1 (cas1) mutant (Babiychuk et al. 2008. Proc Natl Acad Sci USA 105: 3163-3168) and the hmg1;hmg2 double mutant (Suzuki et al. 2009. J Exp Bot 60: 2055-2064).

The FM4-64 dye labels very quickly ealy endosome population and could therefore be used to monitor endocytosis in sterol depleted cells with time course experiment for instance.

Author response: We previously published that FM4-64 internalization from the plasma membrane is reduced in the cpi1-1 mutant (Men et al. 2008. Nature Cell Biol 10: 237-244- Fig. 5b) when compared to the wild type (Men et al. 2008, Fig. 5a) and that FM4-64 fluorescence is strongly retained at the plasma membrane in cpi1-1 when compared to the wild-type (Men et al. 2008, Fig. 5c)where also a stronger internalization is observed (Men et al. 2008, Fig. 5a). These effects could be reliably distinguished after120-180 minutes, but we were not able to reproducible resolve a defect on small early endosomal compartments at a very early time point.

An accessory question is why the manuscript title does not refer to sterols and conversely why the objectives of the work at the end of introduction refers only to sterols?

Author response: The manuscript title needed to be kept short, because EMBO J. requires titles to be 100 characters at maximum. Nevertheless, we now inform the reader in the running title about the aspect of sterol dependence (the abstract already stated that the influence of sterols on endocytosis was one of the aspects tested for in this study). In response to this reviewerís comment, we have also rewritten the end of the introduction on page 5 to "Here, we investigate whether sterol composition and components of the endocytic machinery modulate specificity of KNOLLE syntaxin localization during late cytokinesis." Finally, the results section now ends on the analysis of the drp1a;cpi1-1 double mutant suggesting a synergistic interaction between DRP1A and sterols. We hope to have streamlined the manuscript with respect to the reviewers concern.

From the reader point of view, the relationship between sterols and endocytosis remains a bit confused along the manuscript and needs to be clarified.

Author response: See above.

2- The partial colocalization of sterol dye filipin with different endomembrane markers is an important experiment linking sterols to early endosomes. The authors state that there is a sterol gradient throughout the endomembrane system (last sentence p6). I do not think this claim is entirely justified since the data showed that there is a gradient of colocalization between endomembrane markers and filipin. According to the experiment, the different classes of vesicules are either stained or not i.e containing sterol or not. There is no evidence of gradient of lipids per se. The authors need to rephrase their conclusions.

Author response: Thank you for pointing this out. We have omitted this particular sentence, but now include additional electron microscopic data to further support our point (see below).

I am also quite surprised with the figures. It is difficult to conceive that some KNOLLE vesicules do have sterols and some do not. I agree that sterol content might vary among the population of

vesicules (before and after fusion at the plasmamembrane for instance) but it is surprising that 60% of KNOLLE vesicules are not stained with filipin (the highest relative value among markers.

Author response: The co-localisation method used here is highly conservative as it is based on the geometric centre (centrade) of single fluorescing objects. In case the centrades from objects in two channels do not exactly overlap within 200 nm resolution, no co-localisation is scored. We have chosen this stringent approach, previously described by Boutté et al. 2006. (J Cell Sci 119: 1255-1265) to exclude false positive co-localisation. In fact, we may underestimate the "real" co-localisation value, while methods employing pixel overlap on the other hand, commonly lead to an overestimation.

What is known about the binding dynamic and specificty of filipin with sterols?

We have previously demonstrated that filipin acts as a 3 -hydroxysterol-specific fluorescent probe in vitro on dot blots and in situ in Arabidopsis roots prepared for immunolocalization (Grebe et al. 2003. Curr Biol 13: 1378-1387). Filipin only complexes 3 -hydroxysterols such as sitosterol, campesterol, stigmasterol, cholesterol (in animals) and ergosterol (in fungi). The 3 -hydroxy group is specifically required forfilipin-binding since fluorescence is not observed for 3-cholestenone which solely differs by a keto group in position 3 (Grebe et al. 2003). Cholestrol oxidase catalyses conversion from cholesterol to cholestenone and membranes pretreated with cholesterol oxidaes do not display any filipin fluorescence. Similarly, Arabidopsis whole-mounted roots prepared for immunofluorescence and treated with native cholesterol oxidase display no filipin- sterol fluorescence in contrast to roots treated with denatured cholesterol oxidase (Grebe et al. 2003). Hence, the probe highly specifically detects 3 -hydrosysterols in situ in Arabidopsis roots, but does not discriminate e.g. between different sterols such as sitosterol and campesterol

The binding dynamics and specificity of filipin for sterols have been reviewed in detail by Miller 1984 (Cell Biol Int Rep 8: 519-535). We now integrate and cite this paper on page 7. Filipin is being used as a sterol probe in immunofluorescence and electron microscopy in the yeast and animal field for about 30 years now and this review covers many of the aspects considering specificity and binding dynamics.

Such result might very well reflects the low resolution of the colocalization experiment or a bias in filipin and/or KNOLLE staining. It is not clear to me whether the figures relate to 2D analysis or 3D reconstruction. A single 3D analysis with one marker at a better resolution would definetely validate the approach.

Author response: We have clarified that image analysis has been done in 2D (xy) on single optical z sections acquired at 150 nm section thickness in the Supplementary Material and methods on page 4. The approach has been validated and described by Boutte et al. (2006) which we cite. The resolution in the co-localisation experiments is the highest obtainable by conventional confocal laser scanning or two-photon microscopy for this fluorophore combination. An objective lens with an NA 1.4 at 63x was employed at an electronic zoom of about 4 and a pixel size of about 65-70 nm. Thus, 3D analysis would not result in a "better resolution", because the resolution in z, due to the point-spread function of the objective lens, is by far lower than in xy.

We assume the reviewer meant that 3D analysis would add spatial resolution. Higher resolution could theoretically be achieved by acquiring image stacks in 3D followed by deconvolution or, alternatively, by STED microscopy or electron microscopy. Deconvolution of an appropriate number of z-stack sections and STED microscopy cannot be applied in this case at present, because a) we cannot acquire more than three subsequent images in z due to the rapid photobleaching of the filipin-sterol fluorescence and b) STED methods and equipment for co-localisation of an 364 nm excitable fluorophore (such as filipin) and e.g. GFP have, to our knowledge, not been developed.

Nevertheless, filipin can be used to visualize sterol localization at the subcellular level by electron microscopy on ultrathin about 80 nm sections from e.g. Epon- or Spurr-embedded specimen, because filipin-sterol complexes induce 20-30 nm membrane deformations in sterol containing membranes (cf. Grebe et al. 2003; Orci et al. 1981. PNAS 78:293-297). We previously adapted this method to Arabidopsis roots (Grebe et al. 2003). We now include new, additional electron microscopy data on filipin-sterol complexes in dividing root cells of Arabidopsis located at the cell

plate, and membranes in the plane of cell division, at the trans-Golgi network and prevacuolar compartments (new data Figure 4), whereas no filipin-sterol deformations are found at the ER, cis- and medial Golgi cisternae membranes, mitochondria etc. (new data Figure 4).

This data, now integrated in the results section on page 8/9, supports our immunofluorescence analysis at the highest possible resolution that can currently be achieved for sterol visualization in plants. Our finding that filipin-sterol deformations can be observed at the TGN but hardly at all at Golgi stack cisternae is on the one hand consistent with observations in animal cells (Orci et al. 1981) and on the other hand further supports our claim of a graded localization of sterols throughout the endomembrane system.

3- In the same line, the authors stated that KNOLLE doe not seem to be associated with sterol in lipid rafts. I agree with the authors that KNOLLE might interact with sterol outside lipid rafts. However Fig.4q shows some discrepancies between the two fractions R4 and R8. Indeed R8 does not show a relative enrichment in KNOLLE amount but the intensity of the band is clearly much higher compared to R4. Since this kind of experiment is difficult to normalize quantitatively, it raises some doubt on the actual interaction between KNOLLE and lipid rafts. If R8 is taken from granted, KNOLLE is clearly in an intermediate binding situation compared to the lipid raft marker PM-ATPase and a non raft protein like SMT1.

Author response: As this reviewer points out correctly, and as we stated in the text on page 10, a slight increase in KNOLLE protein level can be observed on Western-blots from DRM extractions at the higher detergent TX 100 versus membrane protein ration of 8 compared to ratio 4. We could observe this effect in three independent experiments, but KNOLLE was always relatively depleted from the DRM fractions at all detergent ratios when compared to the total membrane and the mock-treated fractions. We cannot explain this effect, at present, but it is very obvious that KNOLLE does not behave like a typical DRM-enriched protein, which would be one biochemical characteristic of proteins enriched in membrane rafts. It is possible that a small portion of KNOLLE is associated with DRMs, but that the majority is not, which may explain such a behavior.

The fact that KNOLLE is not associated with lipid raft but is still altered by sterol depletion raises the question of how KNOLLE and sterols interact? A recent work in yeast (Klemm et al. J. Cell Biol. 2009, 185, 601-12) demonstrated that TGN is a dispatching platform for sterol rich domains (rafts) and their corresponding proteins. If the model applies to plant, one would expect that the absence of KNOLLE from rafts should lead to relative insensitivity of KN dynamics to sterol depletion unless it is indirectly mediated by endocytosis. The fact that sterol depletion alters dynamin subcellular distribution (ref 31) suggests that this might be case and would thus provide a possible answer to my first concern.

Author response: We now refer to the Klemm et al. 2009 paper in the introductory sentence on the DRM isolation in the results section on page 9.

Concerning the reviewer's comment on the paper from Sebastian Bednarek's lab (ref. 31) it should be clarified that the change in sterol composition introduced by application of the inhibitor fenpropimorph in ref. 31 did affect the residence time of DRP1A-GFP and CLC-GFP the plasma membrane. This is exactly what we discuss, namely, that the effect of sterol composition may act on the clathrin/dynamin machinery which, as we show in this manuscript, is cleary required to restrict KNOLLE from the plasma membrane, and which in previous publications has been shown to be sensitive most likely to the change in sterol composition introduced by e.g. fenpropimorph. So, apparently, our statement and discussion agrees with this reviewerís view.

4- The measurement of lateral diffusion of KNOLLE-GFP is a very important experiment since it demonstrates that KNOLLE-GFP diffuses in lateral membranes after cytokinesis and rules out the role of sterols in this process. I am convinced with the FRAP and FLIP experiment which are of high standarts. However the use of KNOLLE-GFP protein fusion raises some concern. Contrary to the endogenous KNOLLE protein, KNOLLE-GFP fusion labels not only the cell plate but also the other plasmamembranes. The authors exploited this property for their diffusion experiment but did not comment the reason of the retention of KN-GFP in plasmamembranes. It can be a major concern since the whole point of the study is to unravel the mechanism restricting KNOLLE to the cell plate. One might very well assume that KN-GFP endocytosis is altered because of the new

properties of the fusion protein. So concluding to the absence of sterol effect in lateral diffusion based on the used of a GFP construct which has different membrane retention properties is very questionable. What is the diffusion rate of other rab markers (rabA2a) or FM4-64 in presence or absence of fen?

Author response:

This suggestion to observe additional markers in wild type and under conditions of altered sterol composition has been helpful. Since the cpi1-1 mutant background provides a more specific tool than fen(propimorph) treatment, because we do not know exactly how many additional enzymes other than CPI1 are targeted by fen(propimorph), as well as what other effects the chemical may have on membrane fluidity by itself, we have performed this analysis in cpi1-1 mutant background. Also, the cpi1-1 mutant displays an even higher level of sterol conversion than achieved by high fenpropimorph concentrations included in the growth medium (cf. He et al, 2003 and Men et al. 2008).

We now include FRAP data for bleaching a 4 micrometre region of the plasma membrane for FM4-64, EGFP-LTI6A, GFP-KNOLLE and PIN2-EGFP fluorescence in both wild-type and cpi1-1 mutant background as new data in Supplementary Figure 4E-P. Note, we were unable to fully bleach all surrounding cells for FM4-64 and EGFP-LTI6A fluorescence except for the plane of cell division and retain a signal at the plane of cell division. This is due to the fact that these two molecules showed very fast lateral diffusion and we substantially lost signal from the plane of cell division during bleaching of the surrounding cells. Therefore, we performed these experiments by bleaching a 4 micrometre region at the lateral membrane which was possible in all cases, allows for the comparison of results between all markers and represents the region in which KNOLLE defects are observed in the cpi1-1 mutant. However, none of the markers displayed enhanced lateral diffusion in the cpi1-1 mutant.

We appreciate this suggestion which is similar to point 3 raised by reviewer 2 and the data have already been discussed in more detail above.

2nd Editorial Decision 04 November 2009

Thank you for sending us your revised manuscript. Our original referees have now seen it again, and you will be pleased to learn that in their view you have addressed all criticisms in a satisfactory manner - even though referee 2 still expresses concerns regarding the mechanistic depth of the study. Given the strong positive vote by the other two referees I have come to the conclusion that the paper will now be publishable in The EMBO Journal and you will receive a formal acceptance letter shortly.

Thank you very much again for considering our journal for publication of your work.

Yours sincerely,

Editor
The EMBO Journal

Referee #1 (Remarks to the Author):

The revised version of the manuscript by Boutteé and co-workers satisfyingly addresses my previous concerns. It seems as if the authors also clarified the points raised by the other two reviewers. I do not have any further criticism. Instead, I congratulate the authors to their great piece of work.

Referee #2 (Remarks to the Author):

This is much improved revised manuscript. Authors have addressed the majority of my concerns. The only concern that remains to be addressed is the mechanistic connection between sterols and endocytosis. As authors point out in their response, it would probably take significant effort to elucidate the machinery by which sterols regulate endocytosis, as well as, how endocytosis actually regulate KNOLLE distribution. However, for manuscript to be published at EMBO J., I would expect insight in at least some of the mechanism.

Referee #3 (Remarks to the Author):

The revised manuscript of Boutte et al. entitled "Endocytosis Restricts Arabidopsis KNOLLE Syntaxin To The Cell Division Plane During Late Cytokinesis" provides a comprehensive analysis of the role of KNOLLE in cytokinesis. The role of endocytosis and sterols for restricting KNOLLE to the cell plate is now clearly established. All my concerns raised in the previous version of the manuscript have been extensively clarified and the new data provided really strengthens the different claims of the authors. I am sure that this work will make a major contribution in the cell biology field.

